Disease mutations in CMP-sialic acid transporter SLC35A1 result in abnormal α-dystroglycan O-mannosylation, independent from sialic acid

Moniek Riemersma1,2,3, Julia Sandrock1,2, Thomas J. Boltje4, Christian Büll5, Torben Heise4, Angel Ashikov1,2, Gosse J. Adema5, Hans van Bokhoven3,6 and Dirk J. Lefeber1,2,*

1Department of Neurology, 2Translational Metabolic Laboratory, Department of Laboratory Medicine, 3Department of Human Genetics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands, 4Cluster for Molecular Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, 6525 AJ Nijmegen, The Netherlands, 5Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands and 6Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands

*To whom correspondence should be addressed at: Department of Neurology, Translational Metabolic Laboratory, Department of Laboratory Medicine, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: +31(0) 243613953; Email: dirk.lefeber@radboudumc.nl

Abstract

Binding of cellular α-dystroglycan (α-DG) to its extracellular matrix ligands is fully dependent on a unique O-mannose-linked glycan. Disrupted O-mannosylation is the hallmark of the muscular dystrophy-dystroglycanopathy (MDDG) syndromes. SLC35A1, encoding the transporter of cytidine 5′-monophosphate-sialic acid, was recently identified as MDDG candidate gene. This is surprising, since sialic acid itself is dispensable for α-DG-ligand binding. In a novel SLC35A1-deficient cell model, we demonstrated a lack of α-DG O-mannosylation, ligand binding and incorporation of sialic acids. Removal of sialic acids from HAP1 wild-type cells after incorporation or preventing sialylation during synthesis did not affect α-DG O-mannosylation or ligand binding but did affect sialylation. Lentiviral-mediated complementation with the only known disease mutation p.Q101H failed to restore deficient O-mannosylation in SLC35A1 knockout cells and partly restored sialylation. These data indicate a role for SLC35A1 in α-DG O-mannosylation that is distinct from sialic acid metabolism. In addition, human SLC35A1 deficiency can be considered as a combined disorder of α-DG O-mannosylation and sialylation, a novel variant of the MDDG syndromes.

Introduction

α-Dystroglycan (α-DG) is a heavily glycosylated extracellular protein and binds several extracellular matrix ligands, including laminin (1), agrin (2,3), perlecán (4), neurexin (5), pikachurin (6) and slit (7). α-DG shows a peripheral non-covalent binding with transmembrane protein β-dystroglycan (β-DG), that is bound to dystrophin and F-actin (1,8). This complex, also known as the dystrophin-glycoprotein complex (DGC), connects the extracellular matrix to the cytoskeleton. α-DG-ligand binding requires a specific O-mannose-linked glycan. Two types of O-mannose
glycans are described: a tetrasaccharide Neu5Acα2-3Galβ1-4GlcNAcβ1-2-Man-Ser/Thr (9) and a trisaccharide GalNAcβ1-3GlcNAcβ1-4-Man-Ser/Thr (10). The mannose residue of the trisaccharide contains a 6-O-linked phosphate group (10) that is probably bound to an as far not completely known, but crucial part of the laminin-binding glycan epitope, containing xylose and glucuronic acid residues (11,12).

Defective O-mannosylation results in reduced ligand binding and causes muscular dystrophy-dystroglycanopathy (MDDG) syndromes, a group of severe congenital disorders characterized by muscular dystrophy and brain and eye abnormalities (13). The O-mannose ligand-binding structures are also used by Lassa virus for entry into host cells (14). A recent genome-wide screen for genes involved in Lassa virus entry (15) revealed a large set of genes involved in O-glycans. Clinical symptoms included proteinuria, macrocongenital disorder of glycosylation (CDG) type II (16) with reduced sialylation of protein-linked N-glycans and mucin type O-glycans. Clinical symptoms included proteinuria, macrothrombocytopenia and neurological disease with intellectual disability and epilepsy. In this report, we studied the influence of sialic acid on the synthesis of the laminin-binding O-mannose epitope of α-DG and we studied the effect of the p.Q101H disease mutation on sialic acid metabolism and α-DG O-mannosylation.

Results

SCL35A1 acts independently from sialic acid in functional α-DG O-mannosylation

To analyze the role of SCL35A1 in the O-mannosylation of α-DG, we used two independent SCL35A1 knockout HAP1 cell lines that were generated using TALEN technology (15). These SCL35A1-deficient HAP1 cells are suitable for direct analysis of defective O-mannosylation, in contrast to Lec2 SCL35A1-deficient Chinese hamster ovary (CHO) cells, that require LARGE overexpression for detection of α-DG-laminin binding (17). Thus, these cells provide an ideal model system for monitoring simultaneous effects on sialylation and O-mannosylation.

First, we characterized the sialylation status of the HAP1 SCL35A1 knockout cells. Maackia amurenensis lectin II (MALII), recognizing α-2,3-linked sialic acid residues, was able to bind to HAP1 wild-type cells, whereas binding was abolished for both HAP1 knockout cell lines. Similarly, sialidase-treated wild-type HAP1 cells were unable to bind to MALII (Fig. 1A). Moreover, Arachis hypogaea (peanut) lectin (PNA), recognizing the non-sialylated Galβ1-3GalNAc epitope of core 1 mucin O-glycans, showed a lack of binding in the wild-type cells, but in both knockout cell lines and in the sialidase-treated wild-type cells, a clear PNA staining was observed, confirming the loss of terminal sialic acids (Fig. 1A).

To analyze the effect of sialic acid loss on α-DG-ligand binding, expression of the functional O-mannose glycan was analyzed by a laminin overlay assay and by immunoblotting using the IIH6 antibody that recognizes the laminin-binding epitope, and the DAG1 antibody, that recognizes the α-DG and β-DG core proteins. After sialidase treatment, laminin overlay and IIH6 immunostaining showed an even stronger signal as compared with wild-type cells, indicating that the integrity of the ligand-binding epitope was not affected by the lack of sialic acid moieties (Fig. 1B). Remarkably, for both SCL35A1 knockout cell lines no signal was obtained. Analyses using the DAG1 antibody against the α-DG and β-DG non-glycosylated core proteins showed a small shift in molecular weight of α-DG after sialidase treatment, but a considerably lower molecular weight in both knockout cell lines. These results confirm earlier observations that sialic acid is not directly required for laminin binding (17,18).

We hypothesized that sialic acid could play a role during synthesis of the O-mannose glycan in the Golgi apparatus, by acting as a scaffold for docking of α-DG related glycosyltransferases. To study this, we cultured HAP1 cells in the presence of the synthetic fluorinated sialic acid analog (peracetylated 3Fα2-Neu5Acα), known to inhibit sialic acid incorporation by Golgi resident sialyltransferases (19,20). Inhibition of sialylation in wild-type HAP1 cells revealed identical results as for sialidase-treated cells; lower binding of MALII and increased binding of PNA with no effect on α-DG-laminin binding (Fig. 1).

Collectively, these results show that SLC35A1 is essential for synthesis of the functional O-mannose glycan on α-DG, by a mechanism that is independent from sialylation.

Disease causing p.Q101H mutation in SCL35A1 reduces α-DG ligand binding

To analyze the effect of the previously described SCL35A1 missense mutation on α-DG O-mannosylation, we complemented a HAP1 SCL35A1 knockout cell line with wild-type and mutant (p.Q101H) V5-tagged SCL35A1 constructs using lentiviral transduction. Comparable SCL35A1 expression levels were confirmed by anti-V5 immunoblotting (Fig. 2A), and both constructs localized to the Golgi (data not shown) as previously shown in CHO cells (16).

The sialylation levels of the different cell lines were investigated using lectin staining and metabolic labeling. Complementation with wild-type SCL35A1 fully restored the amount of MALII positive cells, while mutant SCL35A1 showed only partial restoration (Fig. 2B and C). In parallel, complementation with wild-type SCL35A1 strongly reduced the amount of PNA positive cells, whereas mutant SCL35A1 complementation resulted only in a slight reduction (Fig. 2B and C).

Metabolic labeling was performed using a novel sialic acid analog, Ac6SiaNPoc, that specifically labels protein- and lipid-linked sialic acids. Cells were incubated with peracetylated Ac5SiaNPoc (Büll, et al., manuscript in preparation) and reacted with a biotinylated-labeled azide, followed by streptavidin-phycocerythin for analysis by flow cytometry. The incorporation of Ac5SiaNPoc was completely absent in the knockout cell lines (Fig. 2D). In knockout cells complemented with a wild-type SCL35A1 construct, Ac6SiaNPoc incorporation was completely restored, whereas complementation with mutant SCL35A1 restored Ac6SiaNPoc incorporation for only ~20% (Fig. 2C). These results confirm the effect of the p.Q101H mutation in SCL35A1 on glycan sialylation as previously shown by mass spectrometry of patient serum N-glycans (16).

Next, expression of the laminin-binding O-mannose glycan was analyzed. In SCL35A1-deficient cells complemented with the wild-type construct, both laminin binding and IIH6 immunoreactivity were restored, but the signal was less intense than for wild-type cells. Possibly less α-DG is present in the sample of the wild-type complemented cells, as also the α-DG core antibody showed a less intense signal. In the mutant complemented cell line, the α-DG core signal was comparable with that of the knockout cell line and both laminin and IIH6 binding were nearly absent (Fig. 2E). These data show that the p.Q101H mutation in SCL35A1 affects not only the transport of CMP-sialic acid, thereby reducing sialic acid levels on glycoproteins, but also the synthesis of the O-mannosyl glycan on α-DG.
Discussion

SLC35A1 is known as a nucleotide sugar transporter that has been considered to be highly selective for CMP-sialic acid (21,22). Although sialic acid is not directly involved in α-DG-laminin binding, as has been shown by sialidase treatment of fully glycosylated α-DG from rabbit skeletal muscle (18), surprisingly a recent Lassa virus screen revealed SLC35A1 as essential gene for functional O-mannosylation of α-DG. Here, we show that SLC35A1 is required for functional O-mannosylation of α-DG, while sialic acid is neither directly nor indirectly needed for the binding of α-DG to laminin.

Previously, Lec2 SLC35A1-deficient CHO cells did not show a defective O-mannosylation in a model where LARGE was overexpressed (17). However, it is known that LARGE overexpression bypasses several O-mannosylation defects (23), and possibly an impairment of SLC35A1 activity on proper synthesis of O-mannose glycans on α-DG may therefore go unnoticed. Here, we employed HAP1 cells for studying sialylation and α-DG-O-mannosylation without the need of LARGE overexpression (17) to reach sufficient expression levels of functional O-mannosylated α-DG. Knockout of SLC35A1 in HAP1 cells clearly showed a defect in sialylation and O-mannosylation, while neither sialidase treatment nor inhibition of the sialylation resulted in defective α-DG-laminin binding. The considerable difference between the molecular weight of α-DG in sialidase-treated or sialylation-inhibited cells and in SLC35A1 knockout cells, indicates that the knockout cells not only lack sialic acids on the O-mannose glycan. Possibly, SLC35A1 could transport, additional to CMP-sialic acid, another nucleotide sugar, that might be incorporated in the O-mannose glycan and might be crucial for binding of α-DG to laminin.

Previously, other nucleotide transporters have been shown to transport multiple nucleotide sugars. SLC25B4 is a UDP-xylolose and UDP-GlcNAc transporter (25) and HFRC1 transports UDP-glucose and UDP-GlcNAc (26). A recent report suggests that for CMP-sialic acid transport by SLC35A1, CMP-recognition is more important than recognition of the sialic acid substrate (27).

Therefore, these symptoms cannot easily be linked to a single pathway and maybe the effect on multiple pathways in brain development and function result in the dominant neurological phenotype of intellectual disability and epilepsy is known in the MDDG syndromes as well as in CDG. The neurological phenotype of intellectual disability and epilepsy is known in the MDDG syndromes as well as in CDG.
phenotype in this patient. The SLC35A1 missense mutation in this adult patient is relatively mild and might therefore not cause specific O-mannosylation characteristics, such as muscular dystrophy. Recently, genetic defects in combined pathways for N-glycosylation and O-mannosylation have been described resulting in human disorders with mixed clinical phenotypes. Thus far, these have been limited to defects in the synthesis of dolichol-P-mannose required for the initial step in O-mannosylation, including defects in DOLK (30), DPM1 (31), DPM2 (32) and DPM3 (33). This study shows that mutations in SLC35A1 can be added to this growing group of combined CDG and MDDG syndromes.

In conclusion, we showed that SLC35A1 has a crucial role in the O-mannosylation pathway without involvement of sialic acid. SLC35A1 defects can be added to the growing group of diseases that show overlap between MDDG syndromes and N-linked CDG. These novel findings are important for elucidation of the O-mannosylation pathway and the identification of the ligand-binding epitope on α-DG.

Materials and Methods

Cell culture

HAP1 cells (34) were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/l-glutamine (Gibco) at 37°C under 5% CO2 atmosphere. SLC35A1 knockout cells (15) were a kind gift from T.R. Brummelkamp (Netherlands Cancer Institute, Amsterdam, the Netherlands).

Inhibition of sialic acid transfer

P-3Fuc-Neu5Ac was synthesized as described before (20). To inhibit sialylation, cells were cultured 3 days in the presence or absence (control) of 100 µM of P-3Fuc-Neu5Ac.

Metabolic labeling of sialoglycans

To assess incorporation of propargyloxycarbonyl sialic acids into cell surface sialoglycans, HAP1 cells were cultured 3 days in the presence or absence (control) of 100 µM Ac5SiaNPoc (Büll et al., manuscript in preparation), washed in 1× PBS and incubated with 100 µl CuAAC reaction buffer (1× PBS containing 250 µM CuSO4, 250 µM l-histidine, 500 µM sodium ascorbate and 100 µM azide-PEG3-biotin) for 30 min at room temperature. Cells were washed in FACS buffer (1× PBS containing 1% FBS and 0.02% sodium azide) and biotinylated glycoconjugates were conjugated for 10 min at 4°C with 2 µg/ml streptavidin–phycoerythrin (BD Pharmingen). Mean fluorescence intensities were measured using a CyAn ADP flow cytometer (BD Biosciences, San Jose, CA) and quantified utilizing FlowJo software (Tree Star...
Inc., Ashland, OR). Azide-PEG3-biotin, sodium 1-ascorbate, l-histidine and copper(II) sulfate pentahydrate were purchased from Sigma-Aldrich (St. Louis, MO).

Complementation of SLC35A1 knockout HAP1 cells

Full-length wild-type and mutant (p.Q101H) SLC35A1 mRNAs were obtained from pYESucFLAG K SLC35A1 vectors [16] by PCR. The coding sequence was cloned in a pLenti6.2/V5-DEST expression vector containing a C-terminal V5-tag, using the Gateway system (Invitrogen). Constructs were verified by sequence analysis. HEK293FT cells were transfected with pLP1, pLP2, pSVS/G and either the SLC35A1 wild-type or mutant vector to produce virus particles according to manufacturer’s protocol (Life Technologies). Viruses were harvested after 72 h. HAP1 KO cells #1 were transduced by incubation with either the SLC35A1 wild-type or mutant viruses in the presence of 6 µg/ml polybrene. After 24 h incubation, virus was removed and medium was refreshed. Next day, 30 µg/ml blasticidin was added to select virus-transduced cells.

Sample preparation for western blotting

Cells were harvested by scraping in buffer containing 0.35 m sucrose, 30 mM Tris and 3 mM EDTA and enriched using Triticum vulgaris (wheat) lectin (WGA)-beads as described previously [30,35]. Subsequent sialidase treatment was performed overnight using 10 mU/µg α(2→3,6,8,9) neuraminidase from Arthrobacter ureafaciens (Sigma-Aldrich) at room temperature. A control reaction was performed without sialidase.

Enrichment of membrane-bound organellar proteins was performed as described previously [36]. In short, HAP1 cells from a confluent 175 cm² flask were harvested by trypsinization. The cell pellet was washed once using PBS and subsequently solubilized in 300 µl digitonin-buffer containing 25 µg/ml digitonin for 10 min. Centrifugation was performed for 10 min at 2000 g at 4°C, supernatant, containing the cytosolic proteins, was removed. The remaining pellet was solubilized in 300 µl 1% Triton X-100 buffer. After centrifugation for 5 min at 7000 g the supernatant was collected and used for western blotting.

Western blotting

WGA-enriched samples were run on a 7.5% homogeneous gel on a PhastSystem gel electrophoresis system (GE Healthcare) following standard protocols and blotted on nitrocellulose (0.2 µm Protein Whatman) by diffusion blotting (60–90 min at 60°C).

Microsomal fractions were run on 10% polyacrylamide gel, either with 4 M Urea (anti-V5) or without (Calnexin). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane by western blotting (Biorad Mini PROTEAN® 3). Lectins and antibodies used: peroxidase-labeled PNA (Sigma-Aldrich, 0.2 µg/ml), biotin-labeled MALII (Vector Labs, 10 µg/ml), anti-αV5 (Life Technologies, 1:5000 dilution), Calnexin (Novus Biologicals, NB100_1965, 1:10000 dilution), IIH6 (Millipore, IIH6C4, 1:2500 dilution), DAG1 (Genetex, GTX105038, 1:333 dilution), β-DG (Novacastra Laboratories, 1:250 dilution). A laminin overlay assay was performed as described previously [30,35].

Lectin flow cytometry analysis

Cells were washed using PBS and subsequently harvested by scraping in cold PBS. Cells were washed twice using blocking buffer (1% Fetal bovine serum, 1 mM CaCl₂ and 1 mM MgCl₂ in PBS). Cells were either incubated with 20 µg/ml tetramethylrhodamine (TRITC)-labeled PNA (Sigma-Aldrich) for 90 min or with 20 µg/ml biotin-labeled MALII (Vector labs) for 45 min. After MALII incubation, cells were washed three times using 1% FBS in PBS and subsequently incubated with 10 µg/ml fluorescein isothiocyanate (FITC)-labeled streptavidin (Sigma-Aldrich) for 10 min. Before measuring, all cells were washed three times using 1% FBS–PBS. The fluorescent signal was measured at a CyAn flow cytometer (Beckman-Coulter). A total of 40 000 cells were analyzed using Summit 4.3 software. Cells incubated with blocking solution or Streptavidin–FITC only were used to determine the background signal. Average of two independent experiments is shown.

Conflict of Interest statement. None declared.

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